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APPEARANCE OF A NOVEL CA^{2+} INFLUX PATHWAY IN SF9 INSECT CELLS FOLLOWING EXPRESSION OF THE TRANSIENT RECEPTOR POTENTIAL-LIKE (*trpl*) PROTEIN OF *DROSOPHILA*

Yanfang HU, Luis VACA, Xi ZHU*, Lutz BIRNBAUMER*, Diana L. KUNZE, and William P. SCHILLING

Depts. of Molecular Physiology & * Biophysics and Cell Biology
Baylor College of Medicine, Houston, TX 77030

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SUMMARY: Activation of phospholipase C, elevation of free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and stimulation of Ca^{2+} influx have been implicated in *Drosophila* phototransduction. Electrophysiological studies suggest that *trp* and *trpl* proteins may be important for the light-activated Ca^{2+} current found in *Drosophila* photoreceptor cells. Although these proteins exhibit homologies to voltage-gated Ca^{2+} and Na^+ channels, their actual function in insect cells and their relation to proteins involved in mammalian cell Ca^{2+} signaling remains unknown. In the present study, $[Ca^{2+}]_i$ was examined in fura-2-loaded Sf9 insect cells infected with recombinant baculovirus containing cDNA for the *trpl* protein. Ca^{2+} influx was examined by use of Ba^{2+}, a Ca^{2+} surrogate that is not a substrate for Ca^{2+}-pumps or carriers and by measurement of whole-cell membrane currents. The results suggest that expression of *trpl* is associated with appearance of a Ca^{2+} permeable, non-selective cation channel formed by the *trpl* protein.

Light stimulation of the photoreceptor cells of *Drosophila* initiates a cascade of events involving activation of phospholipase C, an increase in inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$), mobilization of intracellular Ca^{2+}, and an opening of cation-selective ion channels in the plasmalemma. This causes an increase in membrane current and a sustained depolarization of the receptor potential (1). In the *transient receptor potential* mutant (*trp*), low level light stimulation of the photoreceptor cell produces a near normal response whereas, stimulation with intense light causes only a transient change in receptor potential; the prolonged depolarization seen in the wild type cell is eliminated as is the sustained inward current (2-4). Although the actual function of the *trp* protein has not been determined, it has recently been proposed that *trp* is a light-activated Ca^{2+} channel (3,5). Another protein initially identified as a calmodulin-binding protein, has been cloned from *Drosophila* and designated as *trp*-like or *trpl* since it shares substantial sequence homology with *trp* (5). The proposed transmembrane segments of both *trp* and *trpl* show homologies to membrane spanning regions of voltage-gated Ca^{2+} and Na^+ channels.

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Interestingly, some light-activated membrane current is observed in the *trp* mutant during intense light stimulation although it is only transiently activated (3). This has led to speculation that *trp* encodes for a Ca^{2+}-selective channel responsible for the sustained current component, whereas *trpl* encodes for a Ca^{2+}-activated, non-selective cation channel responsible for the transient change in membrane current (1,5).

In the present study we infected Sf9 insect cells with recombinant baculovirus containing the *trpl* cDNA under control of the polyhedrin promoter (*trpl* cells). Plasmalemmal permeability to Ca^{2+} was determined using both fura-2 and whole cell patch clamp techniques. As control, the results were compared to Sf9 cells infected with recombinant baculovirus containing the cDNA for the M_5 muscarinic receptor (M_5 cells). The results suggest that expression of *trpl* is associated with an increase in plasmalemmal permeability to Ca^{2+} which reflects the activity of a novel cation channel.

MATERIALS AND METHODS

Solutions and reagents. Unless otherwise indicated, MES-buffered saline (MBS) contained the following: 10 mM NaCl, 60 mM KCl, 17 mM $MgCl_2$, 10 mM $CaCl_2$, 4 mM D-glucose, 110 mM sucrose, 0.1% bovine serum albumin, and 10 mM MES, pH adjusted to 6.2 at room temperature with Trizma-base. The full-length cDNA for *trpl* (pAB3.14/Z9) (5) was generously provided by Dr. Leonard E. Kelly (Department of Genetics, University of Melbourne, Parkville, Victoria, Australia).

Culture of Sf9 cells. Sf9 cells were obtained from Invitrogen (San Diego, CA) and were cultured as previously described (6) using Grace's Insect Medium (Gibco) supplemented with lactalbumin hydrolysate, yeastolate, L-glutamine, 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin solution (Gibco).

Production of recombinant baculoviruses and infection of Sf9 cells. The cDNA encoding the M_5 muscarinic receptor and *trpl* were subcloned into baculovirus tranfer vector, pVL1392 and pVL1393, respectively, using standard techniques (7). Recombinant viruses were produced using the BaculoGold Transfection Kit (PharMingen, San Diego, CA). For routine infection, Sf9 cells in Grace's medium were allowed to attach to the bottom of a 100 mm plastic culture dish (10^7 cells/dish). Following incubation for 15 min to 1 hr, an aliquot of viral stock (multiplicity of infection was 20 and 3 for M_5 and *trpl*, respectively) was added and the cultures were maintained at 27°C in a humidified air atmosphere. Unless otherwise indicated, cells were used at 30-36 hrs. post-infection.

Measurement of free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in dispersed Sf9 cells. $[Ca^{2+}]_i$ was measured at room temperature using the fluorescent indicator, fura-2, as previously described (8-10). $[Ca^{2+}]_i$ was calculated by the equation of Grynkiewicz et al. (11) using the K_d value for Ca^{2+} binding to fura-2 of 278 nM determined at 22°C (12). The figures show representative traces from experiments performed at least 3 times.

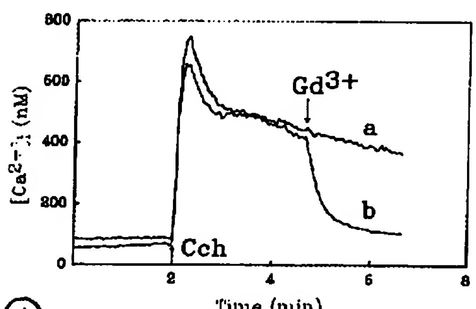
Electrophysiological methods. The patch clamp technique for whole-cell recording was utilized in these studies (13). All electrophysiological experiments were performed at room temperature. Currents were acquired on line and analyzed using the commercially available pClamp programs (Axon Intruments). The pipette (intracellular) solution contained 100 mM Na-Gluconate, 1 mM KCl, 10 mM HEPES, pH. 6.5. Osmolarity was adjusted to 320 mosM with mannitol. The bath (extracellular) solution was MBS.

RESULTS AND DISCUSSION

In order to examine endogenous Ca^{2+} signaling mechanisms, Sf9 cells were infected with recombinant baculovirus containing the cDNA for the M_5 receptor. Addition of carbachol to fura-2-loaded M_5 cells produced the typical biphasic $[Ca^{2+}]_i$ profile commonly seen in non-excitable cells of mammalian origin (Fig. 1). The $[Ca^{2+}]_i$ initially increased 6 to 8-fold over the basal value and subsequently declined with time to a steady elevated phase. Addition of the Ca^{2+} influx blockers, Gd^{3+} (Fig.1; 1 μM) or La^{3+} (10 μM; not shown), during the sustained component of the response to carbachol rapidly returned $[Ca^{2+}]_i$ to the basal level suggesting that the sustained component is dependent upon Ca^{2+} influx. Thus, although the Sf9 cells have an endogenous Ca^{2+} influx pathway that can be activated by stimulation a heterologous membrane receptor, this Ca^{2+} influx is blocked by low concentrations of lanthanides. Carbachol had no effect on uninfected Sf9 cells or on cells infected with baculovirus containing an unrelated cDNA.

In contrast to M_5 infected cells in which the resting $[Ca^{2+}]_i$ was 88 ± 5 nM, basal $[Ca^{2+}]_i$ was significantly ($p<0.001$) increased to 293 ± 21 nM in *trpl* cells examined 30 to 36 hrs post-infection (mean ± S.E. of 7 independent infections). Basal $[Ca^{2+}]_i$ in *trpl* cells was unchanged at 6 and 12 hrs post-infection, but increased in a time-dependent fashion from 24 to 48 hrs (Fig. 2). Thus, the increase in basal $[Ca^{2+}]_i$ in the *trpl* cells occurs over a time frame appropriate for expression of a protein under control of the polyhedrin promoter (14).

The change in basal $[Ca^{2+}]_i$ might reflect either an inhibition of the Ca^{2+} pumping mechanism(s) of the Sf9 cell or an increase in Ca^{2+} influx from the extracellular space. In order to clearly distinguish between these two possibilities we have employed Ba^{2+}. Ba^{2+} has been



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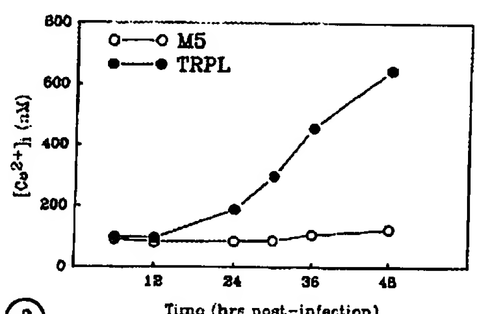

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Fig. 1. Effect of carbachol on $[Ca^{2+}]_i$ in Sf9 cells infected with recombinant baculovirus containing the M_5 muscarinic receptor. Two traces are shown superimposed. Carbachol (Cch; 100 μM) was added to fura-2-loaded Sf9 cells (30-36 hrs post-infection) at the time indicated by the arrow in each trace. $GdCl_3$ (1 μM) was added to one trace during the sustained component of the Cch response (trace b).

Fig. 2. Effect of post-infection time on *trpl*-induced increase in basal $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured in fura-2-loaded Sf9 cells at various times following infection with either M_5 (O) or *trpl* (●) containing baculovirus. Representative results of two independent infections.

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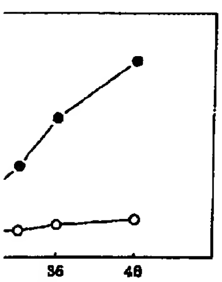

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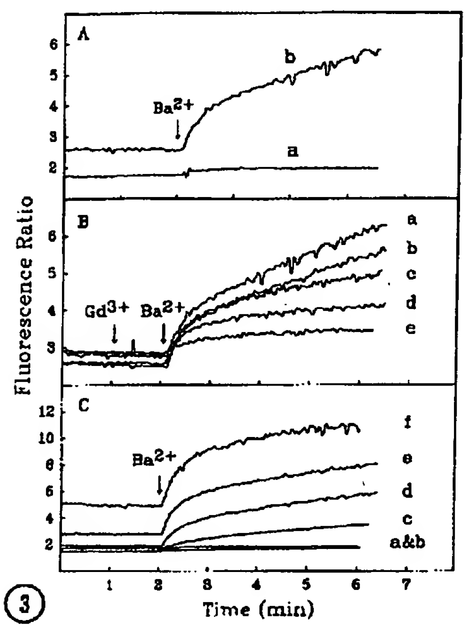
shown to carry current through all known Ca^{2+} channels (15), but is a poor substrate for known Ca^{2+} pumps and transporters (8,16,17). Ba^{2+} will however, bind to fura-2 and change fluorescence in a fashion analogous to Ca^{2+} (8). Addition of Ba^{2+} to M5 cells incubated in Ca^{2+}-free buffer had very little effect on cell fluorescence (Fig. 3A). In contrast, addition of Ba^{2+} to *trpl* cells produced a dramatic increase in fluorescence ratio indicative of Ba^{2+} influx. To confirm that the change in fluorescence following Ba^{2+} addition resulted from influx, Gd^{3+} was added before the addition of Ba^{2+} to *trpl* cells incubated in Ca^{2+}-free buffer (Fig. 3B). Gd^{3+} produced a concentration-dependent inhibition of Ba^{2+} influx in *trpl* cells with an apparent IC_{50} of approximately 200 µM. Basal Ba^{2+} influx was not observed at 6 or 12 hours post-infection,



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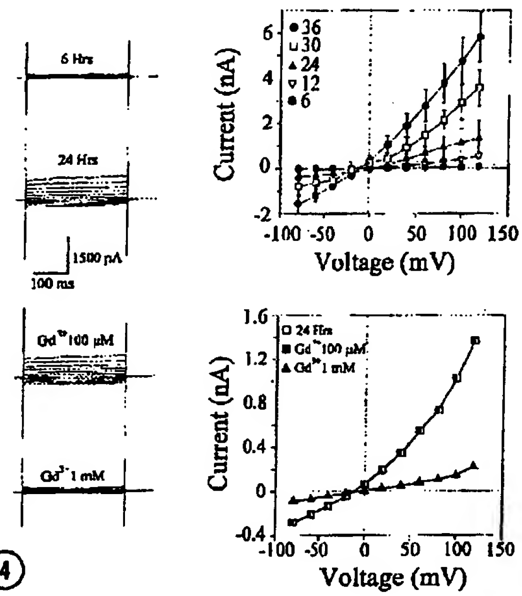

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Fig. 3. Ba^{2+} influx in M_5 and *trpl* infected Sf9 cells. *Panel A:* Fluorescence ratio (for Ba^{2+} excitation wavelength alternated between 350 and 390 nm (8)) was measured in fura-2-loaded Sf9 cells suspended in Ca^{2+}-free MBS (30-36 hrs. post-infection). At the time indicated, $BaCl_2$ (10 mM) was added to either M_5 infected cells (trace a) or *trpl*-infected cells (trace b). *Panel B:* Five traces are superimposed. $BaCl_2$ (10 mM) was added at the time indicated to *trpl* infected Sf9 cells in the absence (trace a) or in the presence of 10, 100, 300, or 1000 µM $GdCl_3$ added at the arrow in traces b-e, respectively. *Panel C:* Cells were incubated in Ca^{2+}-free MBS. Six traces are shown superimposed. Ba^{2+} was added in each trace at the time indicated by the arrow. Traces a, b, c, d, e, and f show the response of the cells to added $BaCl_2$ (10 mM) at post-infection times of 6, 12, 24, 30, 36, and 48 hrs, respectively. Representative results of two independent infections.

Fig. 4. Whole cell current recording in *trpl*-infected Sf9 cells. Traces on the left show current records at different potentials obtained from Sf9 cells at 6 and 24 hours post-infection. The complete I-V is shown on the upper right at the indicated times post-infection. Each value represents the mean ± SD, n=10 cells. The lower two sets of traces were recorded at 24 hrs post-infection in the presence of 0.1 and 1 mM Gd^{3+} in the extracellular buffer. The complete I-V is shown on the lower right. Cell voltage was held at 0 mV and pulses were applied for 300 msec every 2 sec from -80 to +120 mV in 20 mV increments.

but increased in a time-dependent fashion from 24-48 hours (Fig. 3C) which correlates with the change in basal $[Ca^{2+}]_i$ seen in Fig. 2. These results suggest that expression of *trpl* is associated with an increased Ca^{2+} permeability of the Sf9 cell membrane which is, at least in part, responsible for the increased basal $[Ca^{2+}]_i$ observed in these cells.

To determine if expression of *trpl* is associated with the appearance of a novel cation channel, whole-cell membrane currents were recorded in non-infected Sf9 cells (control), and in M_5- and *trpl*-infected cells. As seen in Fig. 4, step changes in membrane potential from -80 to +120 mV produced large step changes in membrane current in *trpl* cells examined at 24 hours post-infection compared to 6 hours post-infection time. The current-voltage relationship at 6 hours was linear with a slope conductance of ~0.5 nS and a reversal potential near 0 mV. The current observed in *trpl* cells at 6 hours post-infection was not significantly different from control or M5-infected cells examined at 36 hours post-infection. However, current increased in *trpl* cells in a time-dependent fashion from 12 to 36 hours (Fig. 4) reaching a slope conductance of ~50 nS at 36 hours. The whole cell current was unaffected by 100 μM Gd^{3+}, but was reduced to control levels by 1 mM Gd^{3+}. Thus, the sensitivity of this current to Gd^{3+} and the time course of expression are similar to the results of the Ba^{2+} influx experiments.

The current observed in *trpl* cells reversed near zero mV suggesting that the current is cation selective since the equilibrium potential for Cl^- is -120 mV under these ionic conditions. Furthermore, replacing the cations in the extracellular solution with N-methyl-D-glucamine while maintaining Cl^- constant produced a shift in the reversal potential to -100 mV, as expected for a cation channel with low conductance to NMDG. In similar experiments, replacement of the bath solution with Ca^{2+}-gluconate (50 mM) produced little change in the reversal potential (n=5) indicating that the channel has similar permeability to both Na^+ and Ca^{2+}. These results demonstrate that expression of *trpl* is associated with the appearance of a non-selective cation current that also allows permeation of Ca^{2+} into the cell.

There are two possible mechanisms by which expression of the *trpl* protein can increase basal Ca^{2+} influx and membrane current. First, *trpl* may activate an endogenous non-selective cation channel. Or, second, *trpl* may itself form channels in the plasmalemma consistent with its proposed role in *Drosophila* phototransduction. It is clear that both non-infected Sf9 cells and M_5 cells exhibit little whole cell membrane current and there is no evidence for the presence of voltage-gated channel in these cells consistent with a previous report (18). Furthermore, the *trpl*-induced membrane current is large in magnitude, appears in a time-dependent fashion, and shows no evidence of saturation out to 36 hours. Thus, it seems unlikely that *trpl* is activating some endogenous channel pool. The results shown in Fig. 1 demonstrate however, that the Sf9 cell does possess a Ca^{2+} influx pathway that can be activated by heterologous receptor stimulation. The striking feature of this response is the potency of Gd^{3+} for inhibition of Ca^{2+} influx. Whereas 1 μM Gd^{3+} produced complete block of carbachol-induced Ca^{2+} influx, this concentration had essentially no effect on basal Ba^{2+} influx observed in *trpl* cells. Although these

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results may reflect high expression of *trpl* protein, the >1000-fold difference in sensitivity to Gd^{3+} suggests independent pathways. Definitive proof that the *trpl* protein forms a channel will require functional expression and characterization of *trpl* mutants.

In conclusion, the results of the present study are consistent with the hypothesis that *trpl*, and perhaps *trp* proteins, form Ca^{2+} permeable, cation channels. Although of obvious importance for understanding the phototransduction pathway in *Drosophila*, these results may also provide general insight into agonist-induced Ca^{2+} signaling mechanisms in non-excitable cells of mammalian origin. The molecular mechanisms by which depletion of the Ins(1,4,5)P_3-sensitive internal Ca^{2+} store activates a surface membrane Ca^{2+} channel is currently an area of intense research. Although membrane current associated with Ca^{2+} store depletion, designated I_{CRAC}, has been measured in vascular endothelial cells (19), T lymphocytes (20), and in mast cells (21), the single channel events underlying this response have not been recorded. Noise analysis suggests that this current reflects the activity of Ca^{2+} channels of very low conductance (<<1 pS) (22). The protein responsible for this current in mammalian non-excitable cells, and in Sf9 cells, may be structurally homologous to *trp* and/or *trpl*. It will be important to begin screening mammalian cell and Sf9 cell cDNA libraries for these homologous sequences.

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